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The disposition and metabolism of 5-methyltetrahydrofolate in man

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A thesis submitted to the Faculty of Medicine
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TABLE OF CONTENTS

TABLE OF CONTENTS (CON'T)

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ABBREVIATIONS

AICAR	-	5-amino-4-imidazolecarboxamide ribonucleotide
dTMP	-	thymidylic acid
dUMP	-	deoxyuridylic acid
DHF	-	dihydrofolic acid
FGAR	-	formylglycinamide ribonucleotide
FIGLU	-	formiminoglutamic acid
5-MeTHF	-	5-methyltetrahydrofolic acid
GAR	-	glycinamide ribonucleotide
IMP	-	inosinic acid
MTX	-	Methotrexate (amethopterin)
p-ABG	-	*para*-aminobenzoylglutamate
THF	-	tetrahydrofolic acid

INTRODUCTION

COENZYME FORMS

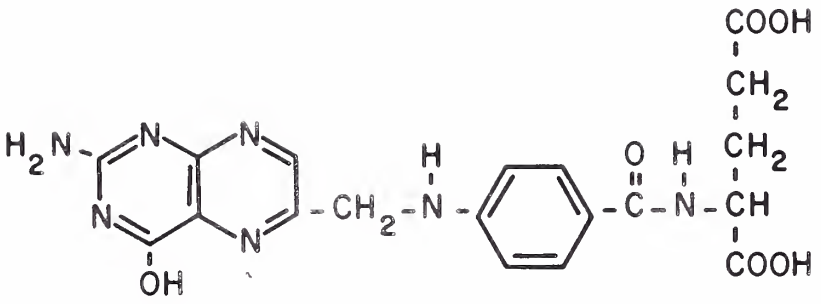
Several coenzyme forms of folic acid (pteroylmonoglutamatic acid) have been described in recent years. These coenzymes are one-carbon adducts of THF, the fully reduced form of folic acid. In each case it has been found that the one-carbon adducts are carried on either the N^5 or N^{10} position, or both, and are at the methyl, formate, or formaldehyde oxidation states. TABLE I summarizes the one-carbon adducts of the various coenzyme forms of folic acid that have been described. Figure 1 compares the structures of the parent compound and one coenzyme form, 5-MeTHF.

These folate coenzymes are involved in a variety of reactions. They are required for the biosynthesis of nucleic acid precursor units, in particular for the synthesis of the purine ring (10-formylTHF and 5,10-methenylTHF) and of thymidylic acid (5,10-methyleneTHF). The parent compound THF participates in the catabolism of histidine and the interconversion of serine and glycine. The 5-MeTHF form is required for the methylation of homocysteine to yield methionine.

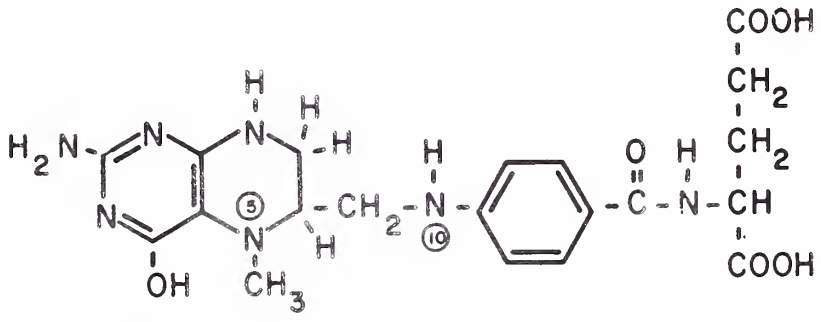
All of the folate coenzymes are interrelated. As each coenzyme donates its one-carbon

TABLE I. The coenzyme forms of folic acid (pteroylmonoglutamic acid) and their one-carbon adducts. (1)

	Adduct	Oxidation State
5-formylTHF	-CHO	formate
10-formylTHF	-CHO	formate
5-forminoTHF	-CH=NH	formate
5,10-methenylTHF	$\geqslant$CH	formate
5,10-methyleneTHF	$>CH_2$	formaldehyde
5-MeTHF	$-CH_3$	methanol



FOLIC ACID (PTEROYLMONOGLUTAMIC ACID)



N^5 METHYL TETRAHYDROFOLIC ACID

Figure 1. Structural relationship between folic acid and 5-MeTHF.

adduct, THF is regenerated in all but one reaction - the biosynthesis of thymidylate from deoxyuridylate. It has been found (2) that the folate coenzyme involved in this last named reaction is 5,10-methyleneTHF. This reaction requires the simultaneous donation of a hydroxymethyl group and of two protons from the THF to form the thymine methyl. Thus, DHF, rather than THF, is produced. The enzyme dihydrofolate reductase then regenerates THF. Alternately, 5,10-methyleneTHF may be converted to 5,10-methenylTHF or 5-MeTHF. The interconversion of the folate coenzymes are presented in Figure 2.

Once the THF molecule has been produced, there are at least three pathways by which it may gain a one-carbon adduct.

The first possibility is associated with the catabolism of histidine. THF may react with the formimino group of FIGLU; this group is transferred to the 5 position of THF as a one-carbon fragment and is then able to enter into the one-carbon pool (3,4). (Equations 1 and 2). Because of the dependency of FIGLU metabolism on available stores of THF, an increase of FIGLU in the urine may be evidence of folate deficiency.

formimino-L-glutamic acid + THF $\rightleftharpoons$ 5-formiminoTHF + L-glutamic acid (1)

5-formiminoTHF $\rightleftharpoons$ 5,10-methenylTHF + NH_3 (2)

A second way in which THF can act as an acceptor for a one-carbon fragment is by transfer of the beta carbon

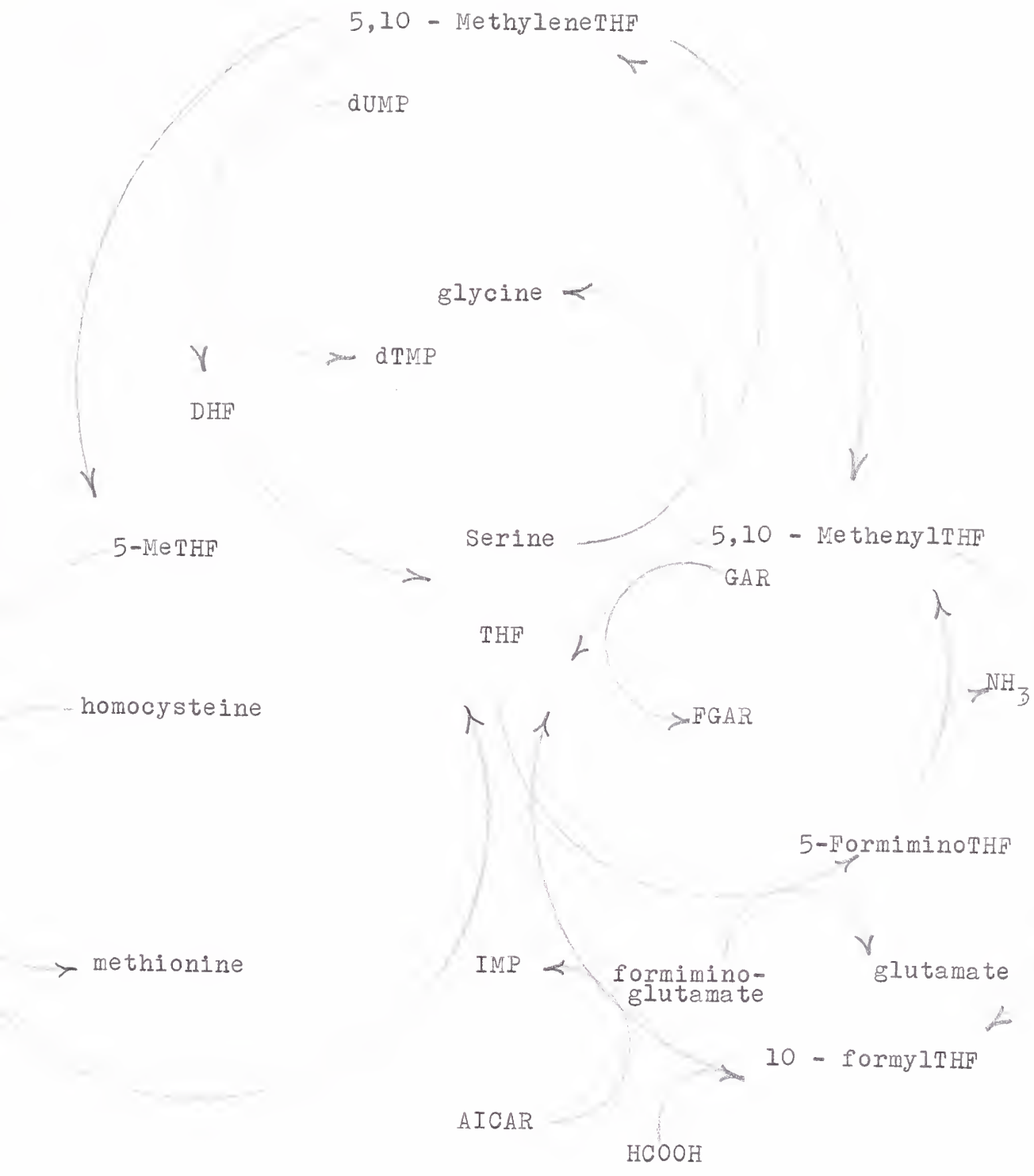


Figure 2. The relationships which exist between the major folate coenzymes in mammalian systems. Modified from Buchanan (5).

of serine to yield 5,10-methyleneTHF and glycine. (Equation 3)

Serine + THF $\rightleftharpoons$ glycine + 5,10-methyleneTHF (3)

The third pathway by which THF may gain a one-carbon adduct is by direct formylation of THF, catalyzed by the enzyme THF formylase. (Equation 4)

HCOOH + ATP + THF $\rightleftharpoons$ 10-formylTHF + ADP + Pi (4)

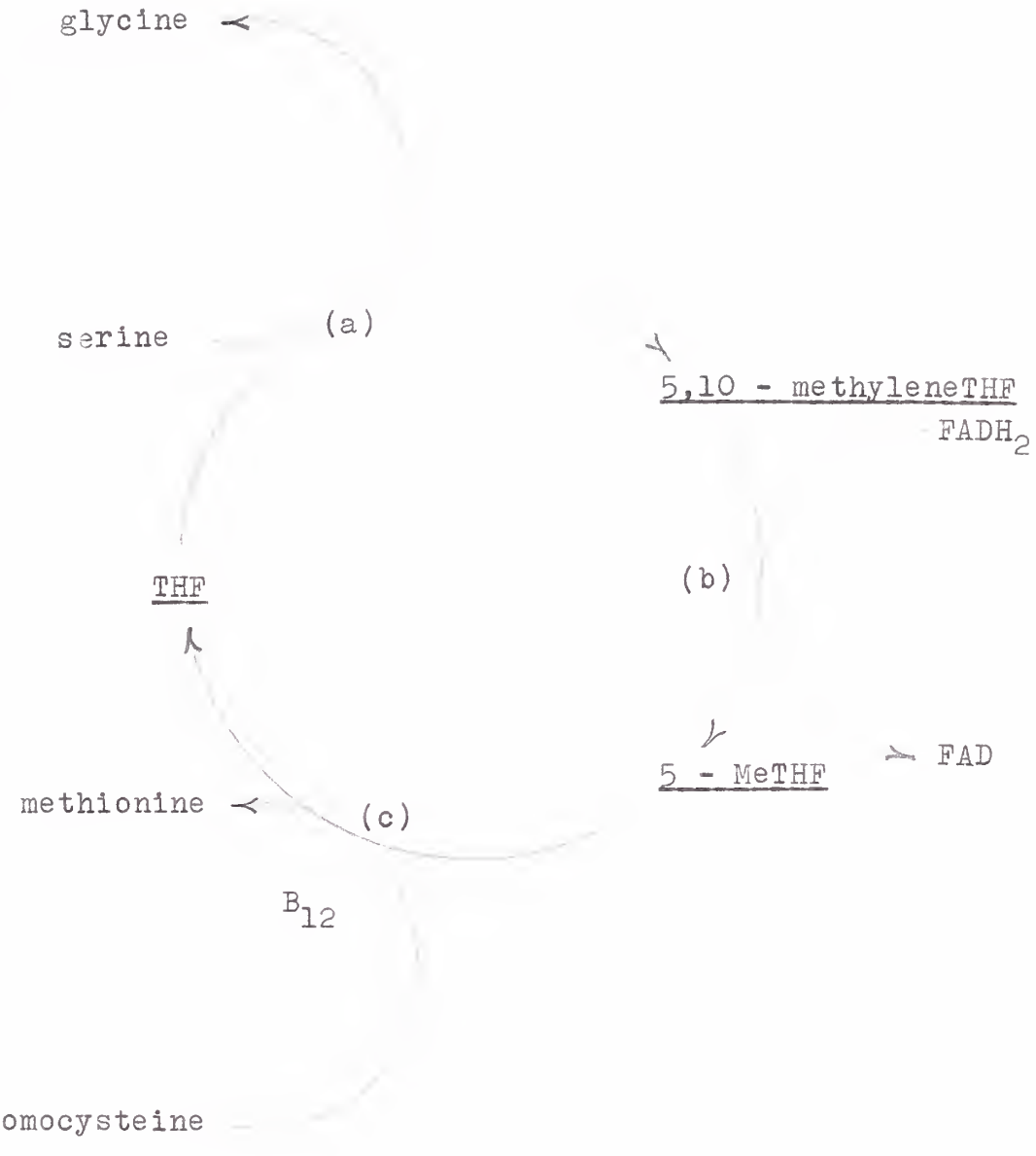
5-MeTHF

Historically, 5-MeTHF, the last folate coenzyme to have been described, was first isolated as "prefolic A" from horse liver (6) and from E. Coli (7); in the latter circumstance as an intermediate in the biosynthesis of methionine from homocysteine. This coenzyme is a major folate compound in the serum (8) and liver (9).

The pathways involved in methionine biosynthesis utilizing 5-MeTHF and vitamin B_{12} are illustrated in Figure 3. Although, in certain bacteria, an alternate pathway for methionine biosynthesis not requiring a vitamin B_{12} coenzyme may exist, in mammalian tissue, evidence indicates that the methylation of homocysteine requires vitamin B_{12} coenzyme as well as 5-MeTHF.

RELATIONSHIP BETWEEN FOLIC ACID, VITAMIN B_{12}, AND MEGALOBLASTOSIS

Clinically a deficiency of either B_{12} or folic acid may produce a megaloblastic anemia. Marshall and Jandl (10) made the important observation that patients with megaloblastic anemia due to folate deficiency



(a) serine hydroxymethyltransferase

(b) 5,10-methyleneTHF reductase

(c) 5-methyltetrahydrofolate-homocysteine methyltransferase B_{12} coenzyme essential cofactor

Figure 3. Relationship of methionine biosynthesis, folic acid, and vitamin B_{12}. Modified from Buchanan (5).

responded to physiologic (0.4 mgm) doses of folic acid, but that B_{12} deficient patients gave no response to this dose. They further noted that B_{12} deficient patients did respond to much greater (pharmacologic) doses of folic acid (15 mgm); folate deficient patients responded as well to the smaller dose as to the larger dose.

The most widely accepted hypothesis for the interrelationships of folic acid and vitamin B_{12} in hemopoiesis is that lack of vitamin B_{12} causes the available folates to become "trapped" as the 5-MeTHF form (11), since 5-MeTHF is able to proceed to THF only after the B_{12}-dependent methylation of homocysteine. Since the equilibrium for the formation of 5-MeTHF from 5,10-methyleneTHF lies far in the direction of the formation of 5-MeTHF, the diversion of large amounts of folates into the 5-MeTHF form would reduce the availability of the other folate forms which are required for the synthesis of nucleic acids. This may explain those clinical signs of folate deficiency which result from B_{12} deficiency.

This hypothesis is not universally accepted. Beck (12) has proposed an alternative hypothesis to explain the megaloblastic hemopoiesis that occurs in B_{12} deficiency. In L. leichmanii coenzyme B_{12} is required for the reduction of ribose nucleotides to deoxyribose nucleotides, the precursors of DNA; however, the enzyme catalyzing this reduction in E. Coli does not require B_{12}. The participation of vitamin B_{12} in this

conversion has not been established for mammalian systems, but Beck proposes that it is required for hemopoiesis and that, under conditions of B_{12} deficiency, limitation of this pathway may be significant for the megaloblastic hemopoiesis.

PLASMA AND URINE FOLATES

Spray et al. (13) noted in 1951 that compared to the situation in normal subjects, there was a more rapid plasma disappearance of exogenously administered folic acid in patients with megaloblastic anemia due to folic deficiency. This rapid disappearance was again noted in 1961 by Metz et al. (14) who also reported a faster plasma disappearance of injected folic acid in vitamin B_{12} deficient patients than in normal subjects.

Later studies (15) suggested two phases of plasma disappearance of folic acid. The first phase could be explained by a rapid uptake by the tissues. It was suggested that the prolonged second phase consisted of removal of folate from the plasma into the urine after the tissues had been saturated.

Johns et al. (16) employing ^{3}H labeled folic acid demonstrated that plasma disappearance was related to the dose. When small doses of folic acid (1 ug/kg) were injected, the plasma disappearance was rapid and urinary excretion was slight. By increasing the dose (to 15 ug/kg and up to 150 ug/kg), the plasma disappearance

was relatively slower and urinary excretion accounted for a much larger percent of the folate removed. With small doses or in folate deficient tissues, tissue uptake plays a much more important role than with larger doses or greater tissue saturation.

By displacement studies with unlabeled folic acid it was also shown that, although folic acid is quickly removed from the plasma and accumulated within the tissues, it is only slowly converted to a non-displaceable form.

Condit and Grob (17) have reported that 99% of endogenous filtered folic and folinic acids are reabsorbed by the kidney; but if the plasma concentration was increased to 100-500 times greater than the physiological level, only 50% of the filtered load was reabsorbed.

Chanarin and Bennett (18) studied the importance of renal clearance for folic acid. Normal patients and patients with impaired renal function were given folic acid intravenously. As expected, those with impaired kidney function had high serum folate levels for longer periods of time than did normals. The same study by Chanarin *et al*. indicated a direct relationship between the serum folic acid level and the renal excretion of folic acid.

Goresky *et al*. (19) substantiated the findings that folic acid is actively reabsorbed from the kidney

tubules and suggested that exogenously administered folic acid is stored within these tubule cells until metabolized. After being metabolized, it is slowly released into the blood. Further, these authors showed that MTX inhibits folic acid reabsorption.

METHOTREXATE (AMETHOPTERIN)

Methotrexate (MTX) is a potent folic acid antagonist which acts by competitive inhibition of the enzyme dihydrofolate reductase. Structurally, MTX is 4-amino-4-deoxy-N^{10}-methyl-folic acid. It has been used successfully in conditions such as acute leukemia in children, choriocarcinoma, mycosis fungoides, psoriasis, and carcinomas of the breast, tongue, pharynx and testes.

Swenseid et al. (20) found that aminopterin increased the urinary excretion of folic acid.

Condit and Grob (17) reported a decrease in the renal tubular reabsorption and decreased extrarenal uptake (normally mainly by the liver) of injected folic acid after aminopterin administration. It appeared as though there was a decrease in the intracellular transport and utilization of folic acid, and that both the renal tubules and the extrarenal sites were affected.

Johns et al. (21)(22) suggested that MTX might compete for the folate transport system. If a patient was preloaded with MTX, the 24 hour urinary

excretion of injected folic acid was about three times greater than in non-preloaded patients. If patients were preloaded with folic acid rather than MTX, similar results were obtained. If the experiment was reversed so that patients were preloaded with labeled folic acid, about seven times more of this label could be flushed out if folic acid was administered rather than MTX. It appeared as if MTX was able to compete with the transport system into cells but was able to enter the cell only slowly if at all.

More recent work (23) again indicated that MTX was only slowly transported into cells and that the fully reduced compounds (tetrahydro) were less effective in displacing MTX than the unreduced (pteroylglutamate) and partially reduced (dihydro) compounds. Of all compounds studied, MTX was the most effective displacing agent for retained MTX.

It has been well known that MTX inhibits the enzyme dihydrofolate reductase by binding very tightly to it (24). More recently, Goldman, et al. (25) have suggested that MTX may also be distributed into two pools other than that bound to dihydrofolate reductase. The first has a low affinity for sites which are located on or near the surface of the cell membrane. The affinity is still present at $0^{\circ}C$, and is weakly inhibited by structural analogues. It is believed not to be related to the transport process.

The second is as a rapidly exchangeable intracellular pool. This pool is believed to exist unbound within the cells. The third and final pool is that which is bound tightly intracellularly to the enzyme dihydrofolate reductase.

Furthermore, it appears as if a transport system exists -- with structural requirements for a substrate similar to but different from the ideal substrate of dihydrofolate reductase. Using Ehrlich ascites carcinoma and L 1210 cells, Kessel and Hall (26) found that folinic acid competed with MTX for transport into these cells.

CHROMATOGRAPHY

It has long been recognized that there are several physiologically-occurring folate coenzymes. Because of the low levels of the coenzymes and the lack of a sensitive chemical assay, in the past these compounds have been identified by microbial assay. The growth requirements of folic acid assay organisms have been reviewed by Stokstad (27), and they are presented in TABLE II.

Although the microbial assay is very sensitive, it is not without problems. The results of the L. casei assay may vary 100 fold depending upon the treatment of the sample (28). The specific factors found to influence the results were the presence of reducing agents (29); the temperature and conditions

TABLE II. Relative activity of various derivatives of folic acid for the support of growth of the commonly-used folic acid assay organisms. (27)

Compound	Streptococcus faecalis (a)	Lactobacillus casei	Pediococcus cerevisiae (Autoclaving Method) (b)	Pediococcus cerevisiae Aseptic (Aseptic Addition)	Tetrahymena geleii
Folic acid (PtGlu)	+	+	-	-	+
Pteroyldiglutamic acid	+	+	-	-	
Pteroyltriglutamic acid	-	+	-	-	+
Pteroylheptaglutamic acid	-	-	-	-	+
H_4PtGlu	+	+	-	+	
5-CHO--H_4PtGlu	+	+	+	+	
10-CHO--H_4PtGlu	+	+	-	+	
5,10-CH=H_4PtGlu	+	+	-	+	
5-CH_3--H_4PtGlu	-	+	-	-	
Pteroic acid	+	-	-	-	-
10-CHO--Pt	+	-	-		
5-CHO--H_4Pt	+	-	-		

(a) Plus indicates activity of 70-100% of folic acid on a molar basis for S.faecalis, Lactobacillus casei, and T.geleii, and 70-100% of 5-formylTH_4 for Leuconostoc citrovorum.

(b) Minus indicates activity of less than 5%.

of storage of the sample (30); the presence of monoglutamate or polyglutamate derivatives (11); and even the disease entity of the patient upon whom the assay is being performed (31). Also, various growth requirements for the bacteria used in the microbial test have been reported within the literature.

The method of identification used in these experiments is column chromatography and is not dependent upon these variables. One of the first attempts at chromatography was by Usdin *et al.* (32). With paper chromatography he was able to separate at least six substances which supported the growth of the assay organisms. All of these had high *L.casei* activity, low *S. faecalis* activity, and even lower *L. citrovorum* activity.

Most of the naturally occuring blood folates later identified by Usdin with TEAE-Cellulose were formylated or reduced and formylated (33). Their number then varied between nine and eleven. The derivative 5-MeTHF was not listed within this group, apparently unknown at that time.

In 1961, Larrabee *et al.* (34) reported a compound in *E. coli* required for the methylation of homocysteine. This compound was identified as 5-MeTHF. Subsequently, Noronha and Silverman (35) reported at least four 5-MeTHF polyglutamates using DEAE-Cellulose.

A similar chromatographic system to that used in these experiments has been found to elute the folate derivatives in the following order: (36)

N^{10} - formylTHF

N^{10} - formylDHF

N^{10} - formyl folic acid

N^{5} - formylTHF

N^{5} - methylTHF

THF

DHF

Folic acid

Pteroyltriglutamate

Chromatography thus enables one to identify an unknown radiolabeled coenzyme by its elution pattern relative to added markers.

PURPOSE

The purpose of these studies was to determine the rate at which physiologic doses of exogenously administered 5-MeTHF is normally metabolized and excreted in man, and how these parameters are affected by a potent antifolate, MTX.

In order to measure the metabolism and disposition of 5-MeTHF, a doubly labeled radioactive molecule of 5-MeTHF was prepared. This molecule was labeled with tritium in the 3' and 5' positions of the benzene ring of the parent molecule and also with carbon-14 at the labile 5-methyl position. In this manner, metabolism of the 5-methyl carbon was followed separately to that of the folate molecule.

The fate of this compound was determined in samples of plasma and urine which were collected at various times after each administration of 5-MeTHF -- both before and after MTX therapy. To identify the (new) folate compounds which were radiolabeled, and to determine the ratio of the radiolabels present in these compounds, representative samples of urine and serum were chromatographed.

METHODS AND MATERIALS

PREPARATION OF DOUBLY-LABELED 5-MeTHF

Preparations of 5-MeTHF were obtained through the courtesy of Dr. P. F. Nixon. The preparations were either unlabeled or radiolabeled with carbon-14 on the 5-methyl group or with tritium at the 3', 5' position of folic acid.

The specific activities of the tritium and carbon-14 were 250 and 12 microcuries per micromole respectively.

The samples were dissolved in 0.7% sodium ascorbate, diluted with physiologic saline, filtered through millipore filters and pyrogen tested.

PATIENTS

Two patients were studied in detail both before and after MTX therapy. Patient 1: 51 year old male suffering from reticulum cell sarcoma of the skin. Patient 2: 56 year old male suffering from carcinoma of the head and neck.

ADMINISTRATION OF 5-MeTHF AND MTX

A dose of 5 microgram/Kg of 5-MeTHF was administered intravenously both before and after MTX therapy. Patient 1: MTX therapy was a 24 hour infusion at 120 mg/meter^2. Readministration of

5-MeTHF followed the completion of MTX therapy by 48 hours. Patient 2: MTX therapy was a 36 hour infusion at 120 mg/meter^2. Readministration of 5-MeTHF followed the completion of MTX therapy by 24 hours.

COLLECTION OF PLASMA, SERUM AND URINE

Blood samples were collected in EDTA. The cells were separated by centrifugation and the plasma was removed and refrigerated.

For serum, large (50-100 ml) blood collections were drawn at the appropriate times, clot formation was allowed, and the serum was recovered after centrifugation. Sufficient 2-mercaptoethanol was added to the serum to achieve a concentration of 0.5 M. The serum samples were then frozen until chromatographed.

Urine samples were collected at intervals and stored in containers. Ten ml of 2-mercaptoethanol were added for the preservation of the reduced folates. The samples were refrigerated until chromatographed.

CHROMATOGRAPHY

Chromatography of samples was through A-25 DEAE-Sephadex (Pharmacia, Inc.) packed in 0.9 x 27 cm columns. Known samples of THF, 5-formylTHF and p-ABG were added to the columns

with the sample to serve as markers. The columns were eluted by potassium phosphate buffer, pH 6.0, the concentration of which was increased in a linear gradient from 0.1 M to 0.8 M, and which was previously gassed with nitrogen and contained 0.2 M 2-mercaptoethanol. The gradient was constructed from 250 ml of each of 0.1 M and 0.8 M solutions arranged so that the concentration began with 0.1 M potassium phosphate and linearly increased until it approached 0.8 M potassium phosphate. Constant volume fractions in the range of 3 to 5 ml were collected. The eluant fractions were analyzed both by fluorometry and by radioactive counting.

Fluorometric analysis was employed because of sensitivity and selectivity. The excitation wavelength was set at 305 mμ, the emission was measured at 360 mμ, and fluorescence was measured at pH 3 and pH 6. At these chosen wavelengths, THF has a greater fluorescence at pH 3 than at pH 6, p-ABG has a slightly greater fluorescence at pH 6 than at pH 3, and 5-formylTHF has slightly greater fluorescence at pH 3 than at pH 6. Since the samples were already at pH 6, after reading at that pH, glacial acetic acid was used to reduce the pH to 3. By combining this analysis with radioactivity counting, the radioactive compounds could be identified by their elution characteristics in reference to the known markers.

The relative positions in which the folate derivatives elute in this system has been determined (37). Figure 4 is an idealized elution pattern obtained from the chromatography of the folate coenzymes.

Chromatography of Serum

After thawing the frozen samples, the supernatant was separated from the gelatinous precipitate and then filtered. Sufficient potassium phosphate pH 6.0 was added to make the filtered supernatant 0.1 M with respect to potassium phosphate, and the sample was chromatographed in the manner described.

Chromatography of Urine

One hundred ml of the refrigerated urine were diluted with an equal volume of water and mixed for one hour with approximately one-third of the A-25 DEAE Sephadex from the column. The Sephadex was previously treated with 0.1 M potassium phosphate buffer pH 6.0 which contained 0.2 M 2-mercaptoethanol. The Sephadex-urine mixture was then poured to complete the top third of a 0.9 x 30 cm column, the bottom two thirds of which was packed with Sephadex unexposed to the urine sample. The sample was then chromatographed in the manner described.

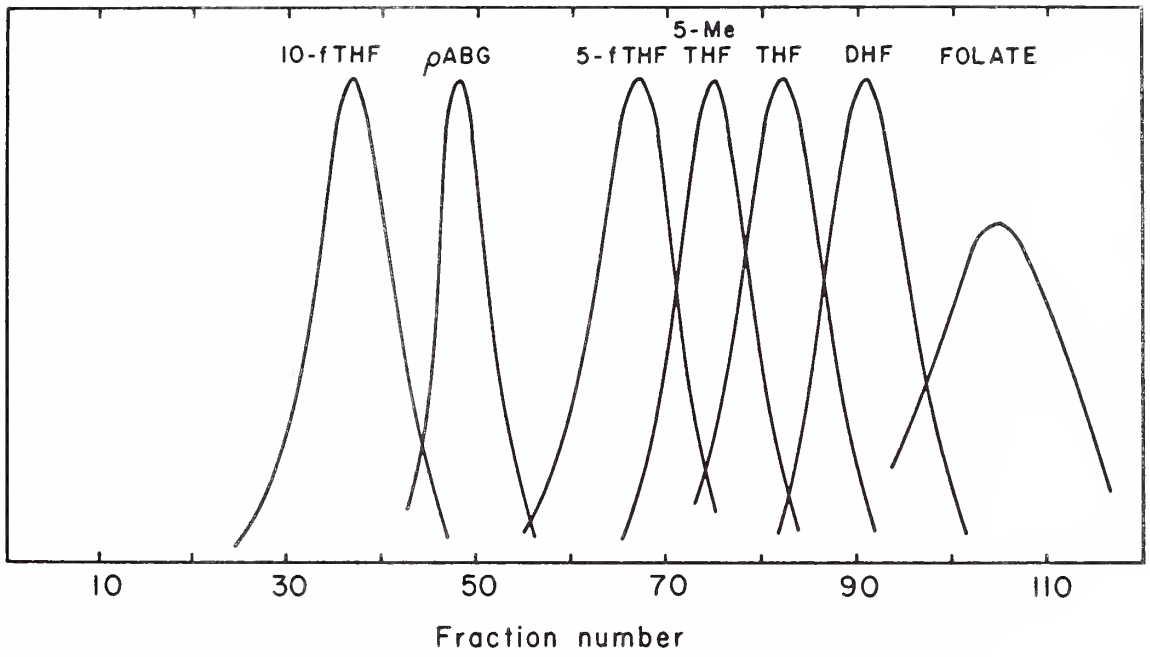


Figure 4. Idealized elution pattern of the folate coenzymes obtained from this chromatographic system.

DETERMINATION OF RADIOACTIVITY

The radioactivity of samples was determined by liquid scintillation counting in a fluid prepared by combining two solutions, one consisting of 100 mgm of POPOP, {p-bis-[2-(5-phenyloxazolyl)] - benzene}, in 2 liters of toluene and the other of 8 Gm of PPO, 2,5 - diphenyloxazole, in one liter of absolute ethanol.

COUNTING

All counting vials were prepared by adding 0.5 ml of the sample to be counted to 15 ml of the counting fluid. Samples were counted for 10 minutes in a Packard Liquid Scintillation Counter. The scalers were set so that one counted carbon-14 but not tritium; the other counted all the tritium plus a constant small fraction of the carbon-14 disintegrations.

Urine

Duplicate 0.5 ml samples were removed from the refrigerated urine and counted as described.

Plasma

Three ml of the refrigerated plasma was added to one ml of 10% perchloric acid and the sample was centrifuged. Duplicate 0.5 ml samples of the supernatant were counted as described.

CALCULATIONS

A sample calculation for the disappearance from the plasma is as follows: the duplicate counts (both in the tritium and carbon channel) were individually averaged and the separate backgrounds subtracted. These values were converted to disintegrations by reference to an External Standardization Channels Ratio count and to a calibrated chart which correlated that Ratio with counting efficiency.

The simultaneous counting of both tritium and carbon-14 resulted in the spillover of a constant fraction of the total carbon-14 counts into the tritium channel. The disintegrations due to tritium were corrected for this spillover. The corrected disintegrations due to each radionuclide were expressed as disintegrations per liter of plasma and in turn, as a percent of the total radioactivity administered in the form of radiolabeled 5-MeTHF.

The calculations involving the urinary excretion were similar. The total disintegrations in the urine sample were determined and expressed as a percent of the total administered radioactivity.

RESULTS

PLASMA

Plasma Disappearance

Plasma disappearances of the radiolabels for the early time periods before and after MTX therapy are illustrated in Figures 5 and 6.

The plasma disappearance appears to consist of two exponential processes--a rapid initial disappearance followed by a longer slower phase. Following MTX therapy, the initial faster phase of plasma disappearance appeared to be shortened. Also after MTX therapy, the plasma levels begin higher and are maintained at higher levels for the first few days, only gradually approaching the levels obtained before therapy.

Before therapy, the percent of carbon-14 of the administered dose reduces to zero at 68 hours and remains there. At 24 hours, the tritium reduces to 0.2% of the administered dose and remains at this level until 114 hours, when the last sample was drawn.

Following MTX therapy, at least 96 hours is required before the carbon-14 level falls to zero. The time required for the tritium to decrease to 0.2% of the administered dose is not greatly affected, occurring at approximately 24 hours.

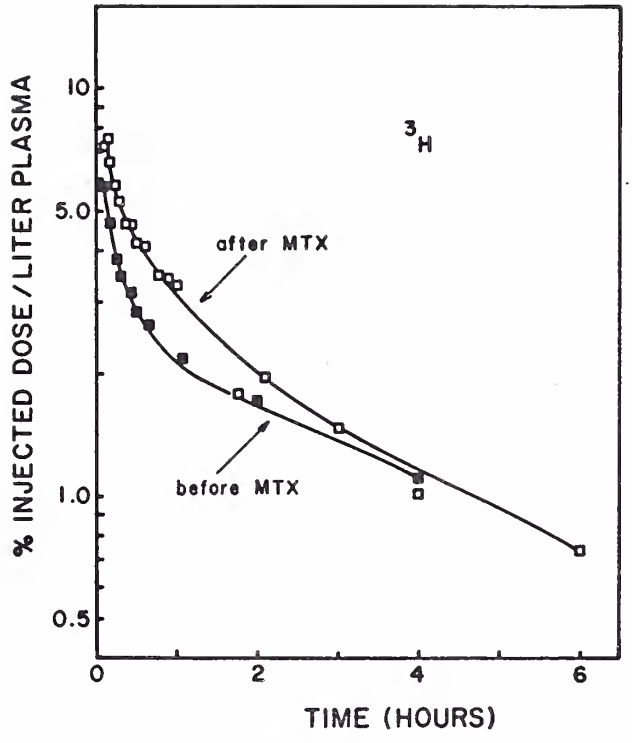


Figure 5.

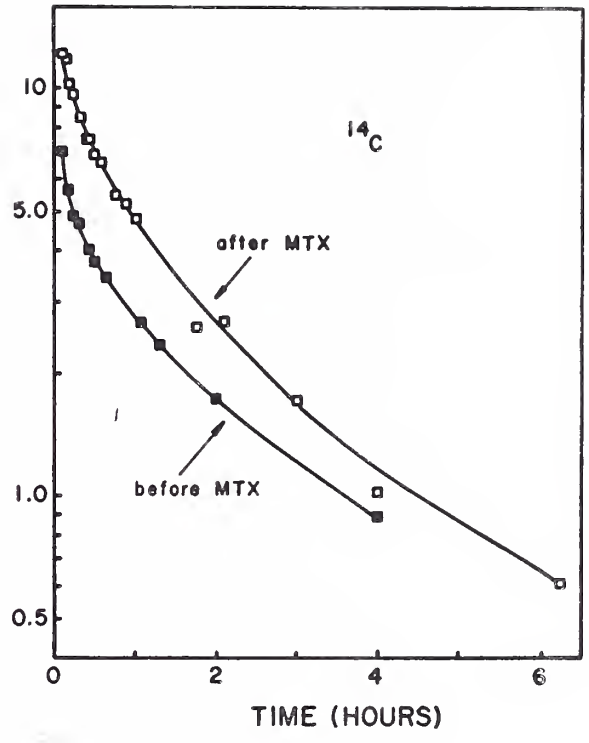


Figure 6.

Figure 5. Plasma disappearance of tritium before and after MTX therapy. (Patient 1).

Figure 6. Plasma disappearance of carbon-14 before and after MTX therapy. (Patient 1).

Ratio of Radiolabels in Plasma

Figure 7 illustrates the ratio of tritium to carbon-14 in the plasma for patient 1 over a 24 hour time period.

After the original administration of 5-MeTHF the plasma ratio ($^{3}H/^{14}C$) was seen to increase greatly with time. In patient 1, the ratio was still increasing at 114 hours and had become so high that it was not possible to measure it. Measurements for patient 2 were terminated at 48 hours, but at that time were increasing similarly to patient 1.

When 5-MeTHF was administered following MTX therapy, the ratio did not begin to increase at once. Rather, an increase was noted but only after 48 hours following the administered dose. At 100 hours, when the studies were concluded, both ratios were still rising.

An increase in the ratio was noted both before and after MTX therapy. Before therapy, it began in a few hours after the administered 5-MeTHF. Following therapy, it was delayed 48 hours.

Chromatography of Serum

TABLE III summarizes the results of the

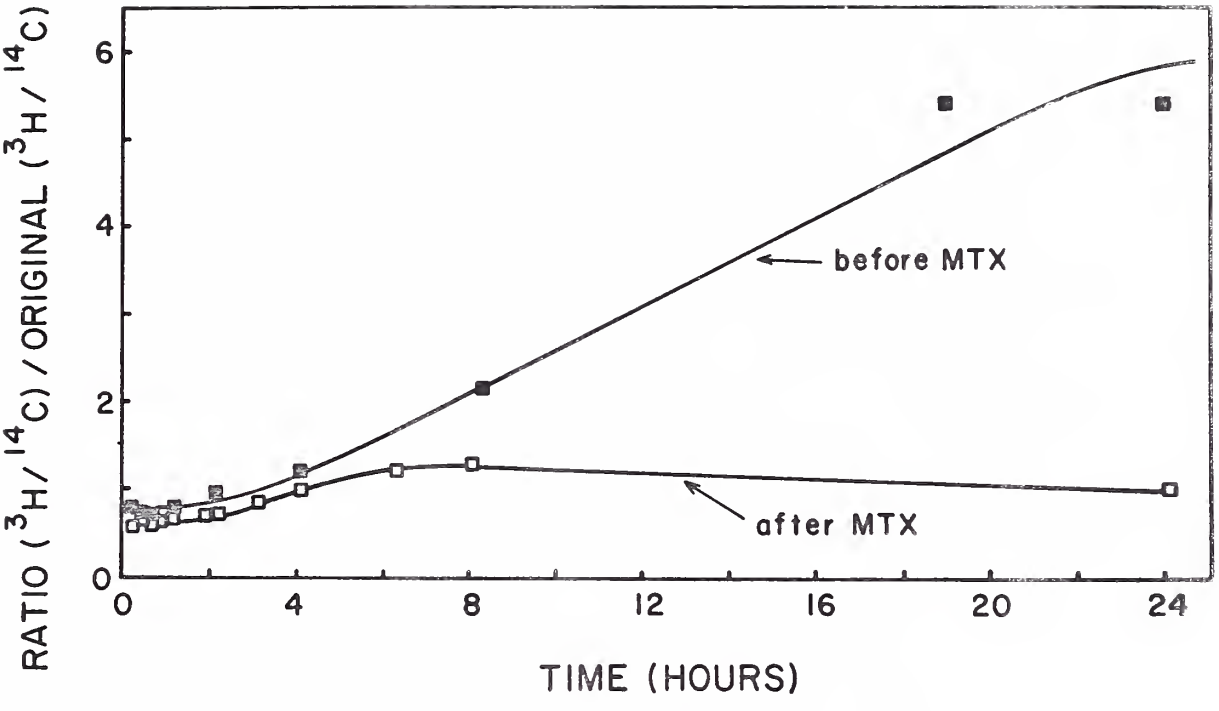


Figure 7. Ratio (^{3}H/^{14}C) of plasma radioactivity divided by the original ratio (^{3}H/^{14}C) of administered radioactivity at the indicated times both before and after MTX therapy. (Patient 1).

TABLE III. Results of the chromatography of serum. The percentages express the ratio of tritium of the particular peak to the total tritium added to the column. The ratios express the ratio of ^{3}H to ^{14}C of that particular peak. The time indicates the interval between the first administered dose of 5-MeTHF and the time of plasma sampling, or the interval between the second administration of 5-MeTHF and the time of serum sampling.

(Patient 1)

		BEFORE MTX	AFTER MTX	
Time		0.5 Hours	0.5 Hours	4 Hours
Original administered ratio ($^{3}H/^{14}C$)		1.8	2.3	
Peak A:	percent	18%	6.9%	1.7%
	ratio $^{3}H/^{14}C$	2.8	1.5	>50
Peak C:	percent	63%	53%	50%
	ratio $^{3}H/^{14}C$	1.9	1.8	5.6

(Patient 2)

		BEFORE MTX	AFTER MTX
Time		4.5 Hours	4.5 Hours
Original administered ratio ($^{3}H/^{14}C$)		17.6	3.62
Peak A:	percent	19%	5.1%
	ratio $^{3}H/^{14}C$	15.9	1.65
Peak C:	percent	98%	50%
	ratio $^{3}H/^{14}C$	26.	43.

chromatography of the serum both before and after MTX therapy.

Two radioactive peaks were consistently obtained from chromatography of the serum. The first peak is unidentified and may be similar to peak A of the urine chromatography (vide infra). Peak C in the serum corresponded to peak C in the urine and has been tentatively identified as 5-MeTHF. The radioactive peak found in urine but not in the serum is thought to be p-ABG, a possible degradation product of THF. The amount of radioactivity found in peak A is decreased after MTX administration and the ratio ($^{3}H/^{14}C$) generally increases with time.

The elution pattern obtained from the chromatography of the serum is similar to the chromatography of the urine except for the p-ABG peak. (See section on the chromatography of urine.)

URINE

Urinary Excretion Rates

Figures 8 and 9 illustrate the urinary excretion rates of the radiolabels either before or after MTX therapy.

Even before MTX therapy, the labels are quickly excreted into the urine. The periods studied often exceeded 100 hours, and within the first 6-8 hours

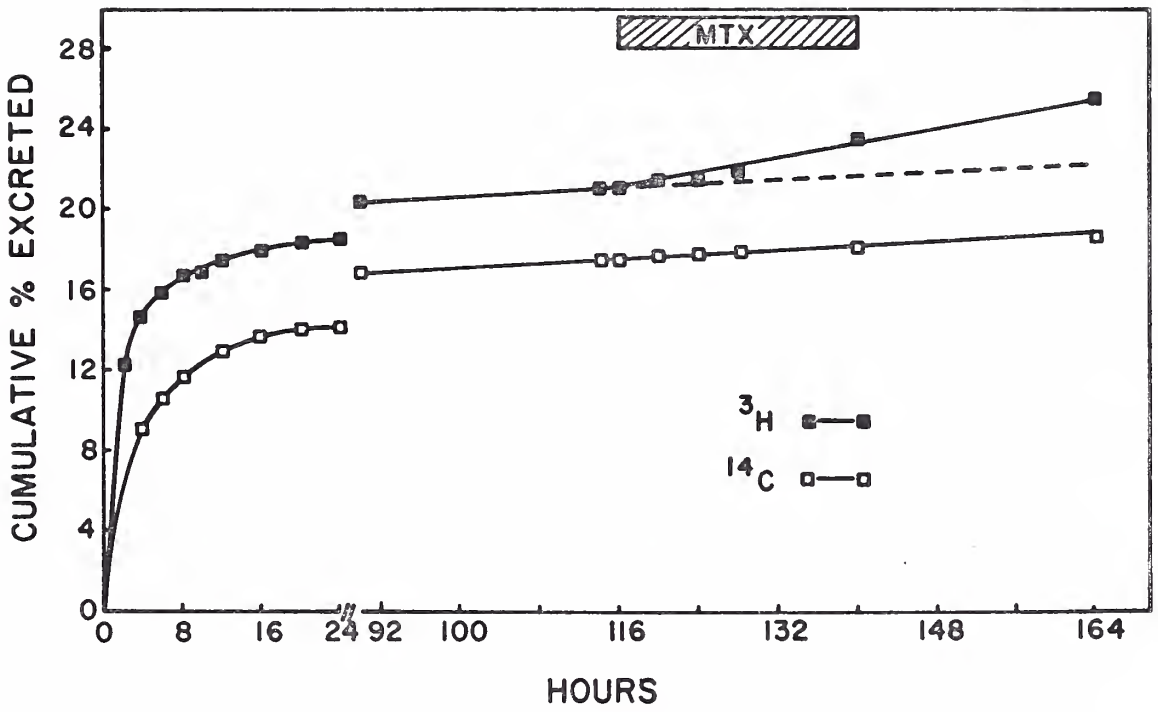


Figure 8. The appearance of radioactivity in the urine. Each label is expressed as a cumulative percent of the administered dose. The period of MTX infusion is indicated. (Patient 1).

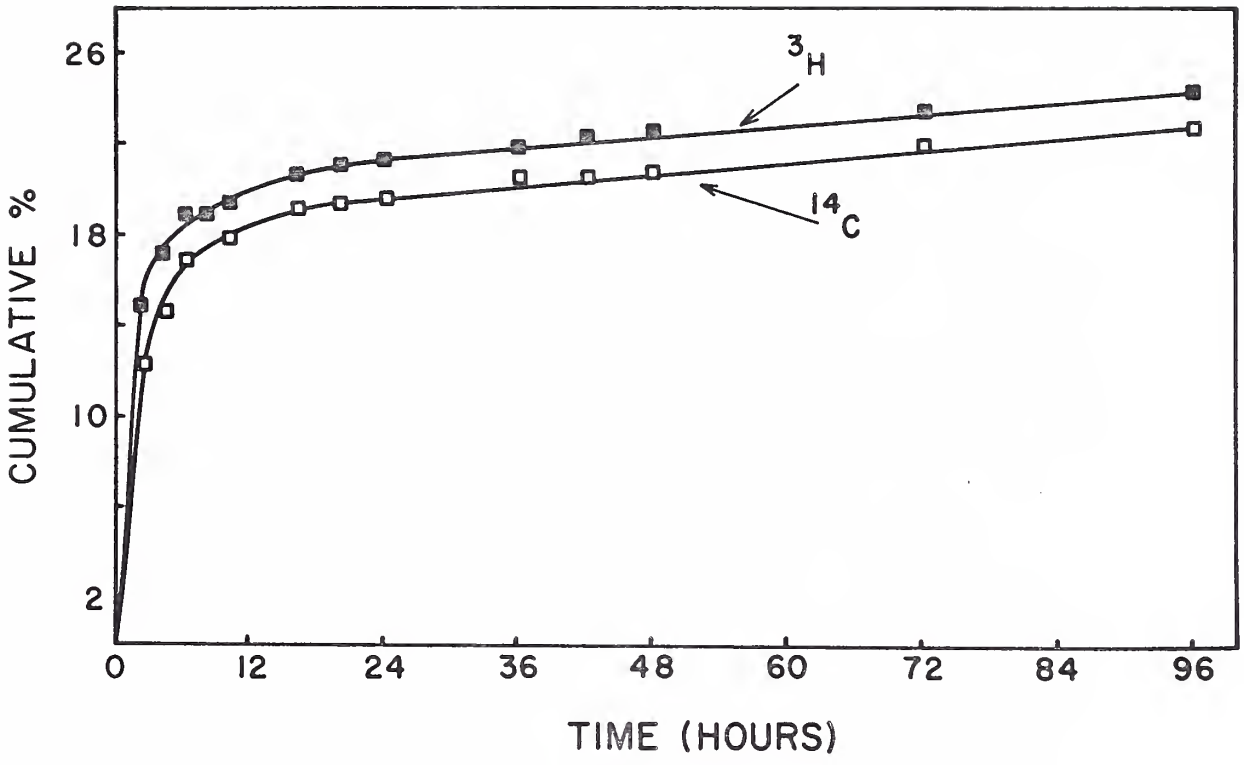


Figure 9. The appearance of the radioactivity in the urine. Each label is expressed as a cumulative percent of the administered dose. The samples were collected following MTX therapy. (Patient 1).

of this period, half of the total excreted radioactivity was already present in the urine. This is indicative of the rapid initial excretion followed by a small percent (less than 2%) being excreted each day thereafter for the duration of the study. This slower, more prolonged rate was constant.

In patient 1, the urinary excretion of the previously administered radiolabels was monitored during a MTX infusion. An increase in the rate of excretion of a tritium labeled compound into the urine was noted (Figure 8). Following this increased initial excretion, the rate of excretion leveled off to less than 2% of the administered dose being excreted each day - similar to the results obtained before MTX.

In both patients, the urinary excretion of the radiolabels was increased after MTX therapy. In patient 1, the increased excretion of radiolabels was less than doubled; but in patient 2, the excretion of radiolabels increased four times. This greater increase could be due to the fact that patient 2 received the second dose of 5-MeTHF only 24 hours after the termination of MTX while patient 1 received the 5-MeTHF 48 hours after MTX therapy.

Of interest, patient 2 excreted a larger percent of carbon-14 both before and after MTX therapy

as compared to patient 1 who excreted a larger percent of tritium. The significance of this is presently unknown.

Ratio of Radiolabels in the Urine

Before the administration of MTX, the ratio ($^{3}H/^{14}C$) remained close to the original administered ratio. Only during the administration of MTX did the ratio increase sharply. These results are illustrated in Figure 10.

Similarly, after the second administration of 5-MeTHF following MTX therapy, the ratio remained close to or slightly less than the originally administered ratio.

Chromatography of the Urine

The results of the chromatography in the urine from patients 1 and 2 both before and after MTX therapy are shown in TABLE IV. Unlike the two peaks obtained from the chromatography in the serum, three peaks were consistently obtained from the urine. The percent of peak A increases with time. Relative to the recovered percentages of the other peaks, peak A tended to decrease after MTX therapy. Before MTX therapy, peak C increases with time and following MTX therapy the percent of peak C in the urine is increased.

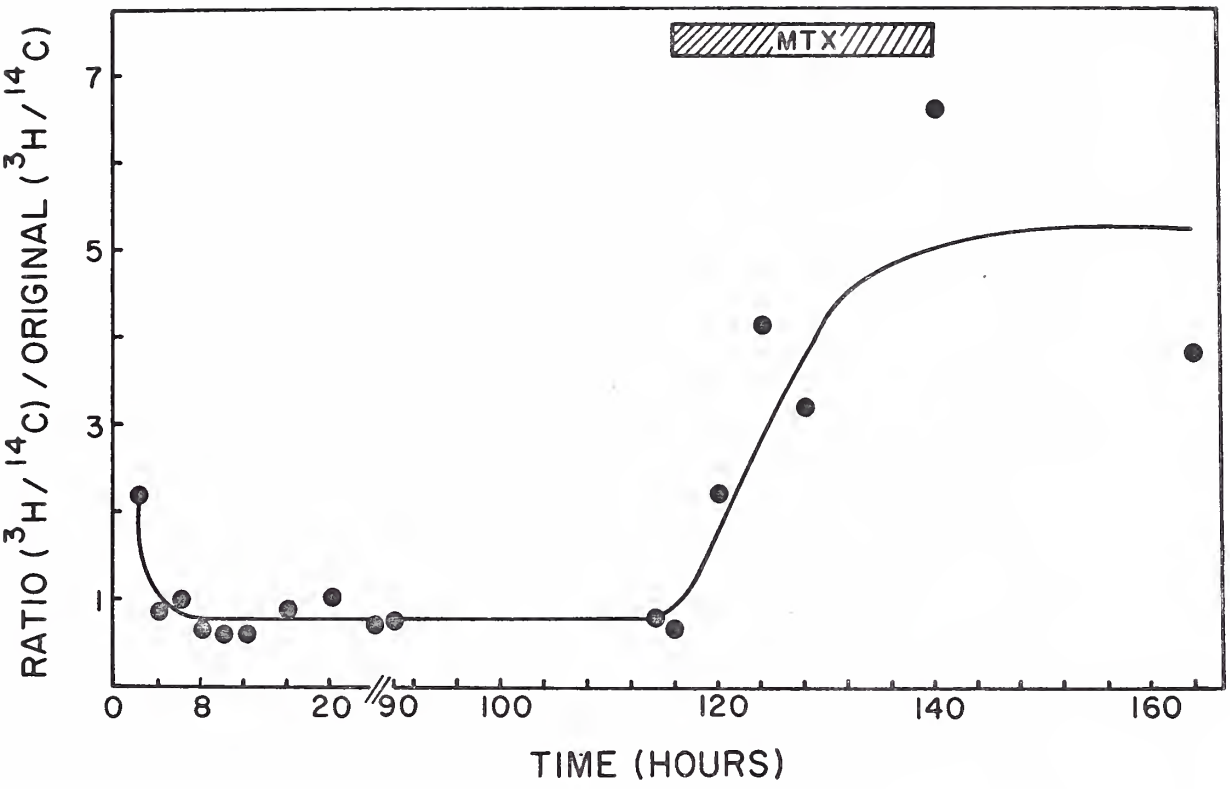


Figure 10. The ratio ($^3H/^{14}C$) of the indicated urine samples divided by the ratio ($^3H/^{14}C$) of the original sample. The time interval is before and during MTX therapy. (Patient 1).

TABLE IV. Results of the chromatography of the representative urine samples. The percents express the ratio of tritium of that particular peak to the total tritium added to the column. The ratio indicates the ratio of ^{3}H to ^{14}C of that particular peak. Time indicates the interval after the original administration of 5-MeTHF and the sample or the interval between the second administration of 5-MeTHF and the sample before or after MTX therapy.

(Patient 1)

		BEFORE MTX		AFTER MTX	
Time		2 Hours	8 Hours	2 Hours	10 Hours
Original administered ratio ($^{3}H/^{14}C$)		1.85		2.35	
Peak A:	percent	1.9%	7.5%	2.7%	8.9%
	ratio $^{3}H/^{14}C$	0.51	0.71	1.3	1.5
Peak B:	percent	10%	12%	12%	21%
	ratio $^{3}H/^{14}C$	6.5	10	6.5	> 50
Peak C:	percent	1.4%	3.3%	39%	15%
	ratio $^{3}H/^{14}C$	1.4	0.45	3.1	5.2

(Patient 2)

		BEFORE MTX			AFTER MTX	
Time		4 Hours	12 Hours	24 Hours	2 Hours	8 Hours
Original administered ratio ($^{3}H/^{14}C$)			17.6		3.62	
Peak A:	percent	1.5%	23%	43%	2.8%	5.0%
	ratio $^{3}H/^{14}C$	7.4	3.6	7.7	2.8	2.0
Peak B:	percent	38%	25%	23%	7.9%	6.1%
	ratio $^{3}H/^{14}C$	> 50	21	12	17	14
Peak C:	percent	11%	22%	28%	21%	47%
	ratio $^{3}H/^{14}C$	18	29	37	3.7	3.4

In each case the sum of these three peaks represented less than the total applied radioactivity. The missing fractions may have remained on the column, may have come straight through the column, may have been degraded to products other than p-ABG, or may have overloaded the column.

Figure 11 is a representative chromatographic elution pattern of a urine sample.

Figure 11. Representative chromatographic elution pattern of the urine. The three peaks are lettered A, B, and C respectively. The shaded areas at the top of the graph represent the elution position of the markers.

Patient 1: 1st two hour urine collection following MTX therapy.

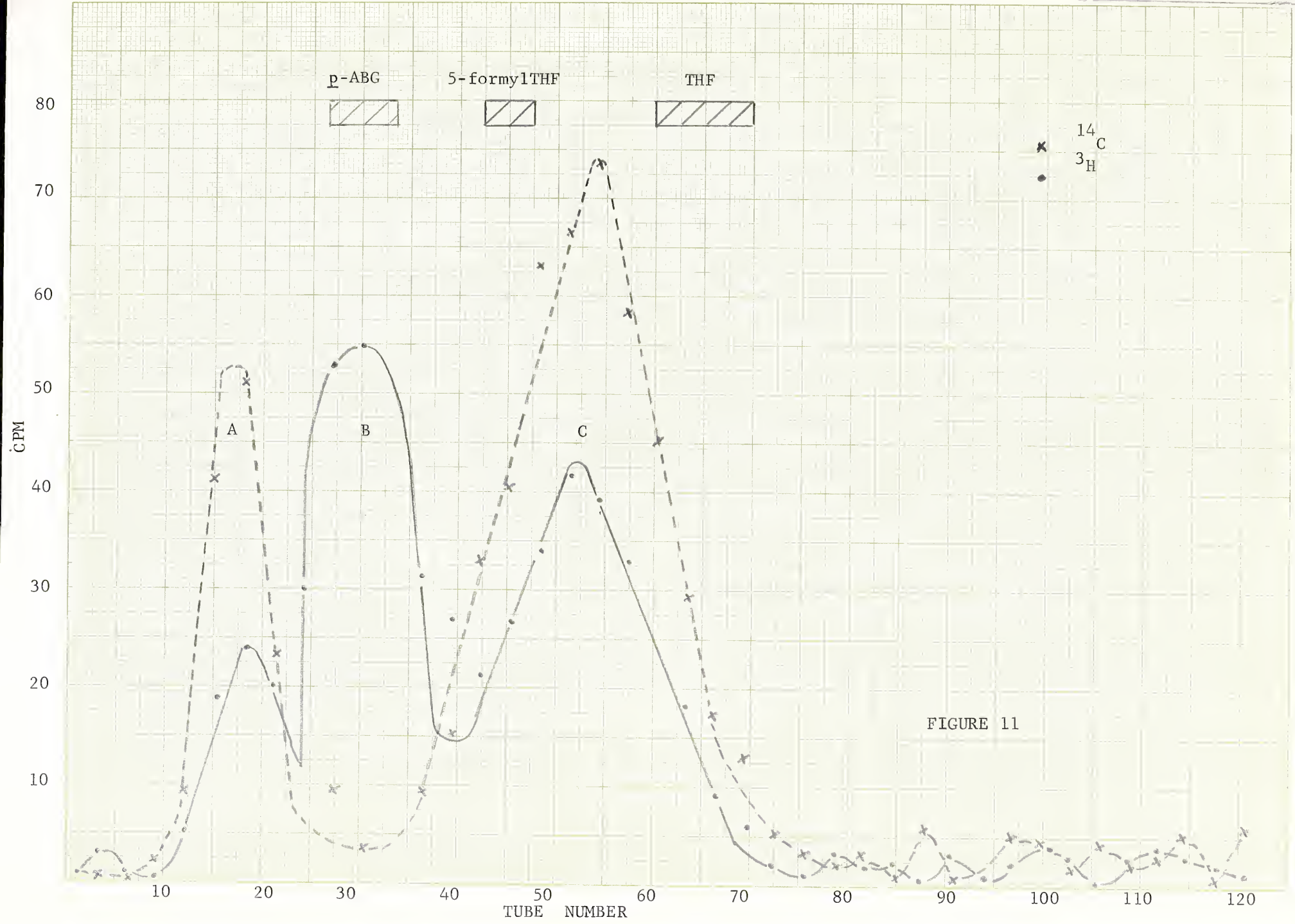

p-ABG
5-formylTHF
THF
^{14}C
^{3}H
A
B
C
CPM
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
110
120
TUBE NUMBER

FIGURE 11

DISCUSSION

PLASMA

The two phase plasma disappearance curves of 5-MeTHF are similar to that reported for folic acid at approximately the same administered dose. The initial rapid phase may be due to uptake of this compound by tissues, mainly liver, kidney and perhaps bone marrow. The slope of the second portion of each of the plasma disappearance curves is less steep than the first portion. The second phase may predominantly represent urinary clearance of this compound and consequently be dependent upon renal function.

In both patients studied, the plasma clearance of radioactivity was slower following MTX therapy. Compared to the levels of radioactivity which were obtained by the administration of 5-MeTHF prior to MTX therapy, plasma levels were initially higher; but after 2 to 3 days, they gradually fell to the same levels. Using the method of Spray *et al.* (38), the physiological distribution of 5-MeTHF was determined from the data both before and after MTX therapy in patient 1 at the two hour period. If MTX does restrict the transport of 5-MeTHF into cells, the distribution of 5-MeTHF should be greater in the absence of MTX; and it is, by 1.4 times.

This data is consistent with the interpretation that the partition of 5-MeTHF out of the extracellular fluid and into the cells is reduced by MTX.

The ratio of plasma ^{3}H radioactivity to ^{14}C radioactivity rose with time in each patient following the first administration of 5-MeTHF. This would be consistent with the belief that the administered 5-MeTHF enters cells and is metabolized; consequently there would be a loss of the ^{14}C-labeled 5-Methyl from the 5-MeTHF. If the parent molecule were to re-enter the plasma, after the loss of the carbon-14, the ratio would increase as it does. This increased ratio indicates that the methyl adduct has been transfered (e.g., to homocysteine to yield methionine) and may have been replaced by another non-labeled one-carbon adduct. This increased ratio was not noted following MTX therapy until 48 hours later and is consistent with the other data which indicates that there is inhibition of cellular uptake of 5-MeTHF by MTX.

Following chromatography of the serum, two radioactive peaks were consistently obtained. The first peak (peak A) contained both ^{3}H and ^{14}C and was eluted earlier than the 10-formylTHF position. It has not yet been identified. The proportion of radioactivity in peak A was less after MTX therapy.

Presumably, after MTX, the administered 5-MeTHF was unable to enter the cells to be converted to this derivative.

The second doubly radiolabeled serum peak (peak C) was eluted at the 5-MeTHF position and almost certainly represented both unchanged 5-MeTHF and also any metabolized coenzyme which had lost the labeled carbon and subsequently gained a non-labeled carbon in the 5 position.

The increased ratio ($^{3}H/^{14}C$) of this peak relative to the administered ratio indicates that the labeled 5-MeTHF group has been replaced to some extent by a non-labeled 5-methyl group. Following MTX therapy, this ratio does not increase.

URINE

Most of the urinary excretion occurred within the first 6-8 hours, a finding which is similar to the data obtained for folic acid (16); however, data comparing identical doses are not available.

The interval between the completion of MTX therapy and the second administration of 5-MeTHF was 24 hours in patient 2 and 48 hours in patient 1; and following MTX the urinary excretion of radio-labels by patient 2 was greater than that by patient 1. From these data, it appears that MTX also exerts an effect on the urinary excretion of 5-MeTHF.

The displacement of tritium into the urine following the administration of MTX is shown in Figure 8. This displacement was not accompanied by a corresponding increase in plasma radioactivity; or should there have been such an increase, it was too small to be detected.

It has been suggested (19) that this increase in urinary radioactivity may represent displaced folates which are being stored within the renal tubules. Unfortunately, the low level of radioactivity prevented identification of this displaced tritium, but there is no detectable increased quantity of urinary carbon-14 simultaneously excreted. This displaced compound could be 5-MeTHF which had been formed *in vivo* from an unlabeled methyl group and tritiated THF; the latter could have resulted from the metabolism of the administered 5-MeTHF. McLean (39) found that following the intravenous administration of tritium labeled folic acid, 25-33% of the total folate displaced was in the form of unlabeled 5-MeTHF. (However, in patients with megaloblastic anemia, some of the 5-MeTHF excreted was labeled indicating a more rapid metabolism in folate deficiency states.)

Three radioactive peaks were obtained from the chromatography of the urine. The first peak (peak A) is believed to be identical with the first serum peak.

The second radioactive peak (peak B) found in the urine was not present in the serum and was tentatively identified as p-ABG or a compound closely related to p-ABG. This identification could be made by the co-chromatography of this radiolabel with known p-ABG and by the absence of ^{14}C from the radioactive peak. It may result from the oxidative degradation of reduced folates following exposure to air (40). Since no radioactivity was associated with the p-ABG peak in the serum, the p-ABG present in the urine samples may have been formed in the urine on standing, despite the addition of 2-mercaptoethanol to the samples. Alternatively, the p-ABG may have been formed *in vivo* prior to excretion, and its excretion may have been very rapid.

The third peak (peak C), tentatively identified as 5-MeTHF, was increased in both patients following MTX. This again indicates that MTX is able to block the availability of serum 5-MeTHF for intracellular metabolism, presumably by blocking its cellular uptake and metabolism.

SUMMARY

The active diastereoisomer of 5-methyl-^{14}C-tetrahydrofolate-3', 5' - ^{3}H has been administered rapidly I.V. at 5 ug/kg to subjects with solid tumors both before and after MTX therapy.

Before MTX, plasma disappearance of both radiolabels was rapid and biphasic. From 10-20% of the radioactivity administered was excreted in the urine within the first 6-8 hours; amounts less than 2% per day were excreted thereafter. Some 24-48 hours following cessation of MTX infusion therapy, plasma disappearance was slower and urinary excretion was greater than before therapy.

Chromatography of serum obtained at 30 minutes and at 4 hours after 5-MeTHF administration showed that greater than 50% of the radioactivity was unchanged 5-MeTHF. Chromatography of urine at 2 and 4 hours showed that less than one half of the radioactivity excreted in the urine was 5-MeTHF, probably due to its breakdown in the urine since a large proportion of the urinary radioactivity was identified as the breakdown product p-ABG. Following MTX therapy a larger proportion of the radioactivity excreted in the urine was identified as 5-MeTHF.

An unidentified doubly labeled derivative was found in both serum and urine. The data showed that its formation was increased with time and inhibited by MTX.

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